Title: Effects of several inocula on the biochemical hydrogen potential of sludge-vinasse co-digestion

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Abstract

The influence of the inoculum on the Biochemical Hydrogen Potential test (BHP) was investigated. Thermophilic BHP from sludge-vinasses co-digestion (50:50) was studied employing three types of inocula: Acidogenic Inoculum, Sludge Inoculum and Thermal Sludge Inoculum. The maximum hydrogen yield was obtained with a sludge inoculum (177mL H_2/g VS_{added}). This yield was 21 and 36% higher than for acidogenic inoculum and thermal sludge inoculum, respectively. The percentages between Eubacteria:Archaea increased from 59.2:40.8 to 92.0:9.0 during BHP tests using the sludge inoculum while it remained stablish in the others cases around 50:50. Furthermore, hydrogen production was accompanied by the generation of volatile fatty acids, mainly acetic, butyric and propionic acids. There were no differences in the rate of hydrogen production in any of the BHP.

Keywords: Biochemical hydrogen potential; Inoculum; Sludge; Vinasse; Anaerobic co-digestion; *Eubacteria*

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1. Introduction

In recent years, the energy crisis has imposed the necessity to achieve a sustainable future built on alternative sources of energy and materials. Molecular hydrogen represents a storable form of energy [1]. Moreover, its combustion does not generate polluting products and it has high specific energy [2–4].

Hydrogen production can occur during the anaerobic digestion (AD) process. This process can be divided into two stages: dark fermentation (DF) and methanogenesis. The first stage involves the production of volatile fatty acids (VFAs), H_2 and CO_2, while the second one converts VFAs into CH_4 and CO_2 [5,6]. Simple operation conditions, low operating cost, low energy demand and fast reaction rate are some one-off advantages of dark fermentation [7]. Hydrogen generation using the DF process is possible with a wide range of waste materials such as sludge [8], food waste [9], cheese whey [10], algal biomass [11] and vinasse [12]. Recently, numerous studies have found that co-digestion of two or more substrates can increase the load of biodegradable organic matter, improve the balance of nutrients, improve microbial diversity leading to enhance hydrogen production [13,14]. Although there are numerous studies on hydrogen production by co-digestion of sludge with different substrates such as perennial ryegrass [2], food waste [15] and glycerol [16], no prior studies have been published on the production of hydrogen via sludge-vinasse co-digestion.

Vinasse is an effluent generated during the production of alcohol in the wine distillation process. This effluent can be highly damaging in the areas in which it is discarded due to its high organic load, low pH and high corrosivity. Instead of harmful, vinasse may be considered as a substrate for hydrogen generation through the dark fermentation process because of the surplus organic load.

47 Biochemical hydrogen potential (BHP) corresponds to the maximum hydrogen
48 production at dark fermentation infinite time and is a key parameter to evaluate the
49 suitability of substrates to obtain biohydrogen. Batch methods have recently been
50 applied to evaluate the BHP of numerous substrates, although the operating conditions
51 (such as pH, temperature) have yet to be standardized. Moreover, there is no consensus
52 regarding the nature of the inoculum to use in these tests or the type of pre-treatment
53 they should receive (Table 1). One of the most widely used types of inoculum is the
54 anaerobic sludge, though from different sources such as municipal sewage [17,18],
55 wastewater [2], poultry slaughterhouse wastewater [19,20] and citrate-producing
56 wastewater have also been used.

57 Most research studies use inocula subjected to thermal pre-treatment in order to
58 enrich the inoculum in terms of the hydrogen-producing bacteria. The pre-treatment is
59 generally carried out at a temperature of 100°C [2,17,18,21,22], although it has been
60 carried out at 90°C in other cases [19,20,23] or even at a temperature above 100°C
61 [24,25]. The exposure times of the inoculum to thermal shock vary greatly, ranging
62 from 15 to 30 minutes in most cases [2,17,18]. However, in the studies by Giordano et
63 al. [25] and Mohan et al. [22], the exposure time was longer (2-4 hours). Other authors
64 use a hydrogen-producing inoculum [3,12,26]. The results of these studies are
65 inconclusive; hence the lack of consensus regarding the type of inoculum or the thermal
66 pre-treatment conditions to be employed in BHP tests.

67 In this study, BHP tests with different natural inocula and pre-treatment conditions
68 were carried out to study their influence on BHP results. The main purpose of this
69 research is to discern which type of inoculum to use for future BHP tests.

70

71 ## 2. Materials and methods

72 ### *2.1. Substrates*

73 Waste activated sludge (WAS) and vinasse (V) were used as substrates. The
74 WAS was collected from Guadalete municipal wastewater treatment plant, Jerez de la
75 Frontera, Cadiz, Spain. The V was provided by the González Byass winery located in
76 Jerez de la Frontera, Cadiz, Spain, and kept frozen (-20°C) until use.

77 A mixture of both substrates in a 50:50 ratio was used as the feedstock in all the
78 BHP tests.

79 ### *2.2. Inocula*

80 Three types of inocula were used: Acidogenic Inoculum (AI), Sludge Inoculum
81 (SI) and Thermal Sludge Inoculum (TSI). The AI was collected from a laboratory scale
82 semi-continuous acidogenic thermophilic anaerobic digester treating waste activated
83 sludge-vinasse (50:50) for hydrogen production. The reactor operated at pH 5.5, a
84 temperature of 55°C and a HRT of 4 days. The AI was thus already conditioned to treat
85 the mixture of WAS-vinasse co-substrates and is, therefore, a hydrogen -producing
86 inoculum. The SI and TSI were collected from a laboratory scale semi-continuous
87 thermophilic anaerobic digester treating waste activated sludge operating at pH 7.0, a
88 temperature of 55 °C and a HRT of 20 days. The TSI was heat-treated in a hot oven at
89 100°C for 15 min.

90 Three BHP tests were carried out, Tests 1, 2 and 3, with the aforementioned
91 inocula, AI, SI and TSI, respectively.

92 The physic-chemical characteristics of the inocula and substrates are
93 summarized in Table 2.

94 Table 2. Physico-chemical characteristics of the inocula and substrates

Parameters	Units	AI	SI	TSI	WAS+V
pH		5.32	5.49	5.52	5.39
TS	g/L	28.68	40.71	38.01	41.07
VS	g/L	21.50	31.85	29.39	33.51
TCOD	g/L	51.81	49.51	42.27	63.75
SCOD	g/L	37.52	22.58	22.62	28.06
Total VFA	g/L	4.93	2.67	3.41	2.14

95

96 *2.3.Biochemical hydrogen potential*

97 Hydrogen fermentation was performed in 250mL glass bottles with a 120mL
98 working volume and a 130mL headspace volume. For each reactor, a mixing ratio of
99 inoculum to feedstock of 1:1 (v/v) was used. The initial pH of each bottle was set at 5.5,
100 a value at which methanogenic *Archaea* are inhibited. Nitrogen was fluxed for 5 min to
101 displace any air present in the bottles and hence ensure an anaerobic environment. All
102 the bottles were maintained at constant temperature under thermophilic conditions
103 (55°C) in an orbital shaker incubator.

104 All the experiments were carried out in triplicate and inoculum control bottles
105 were also prepared. Three bottles were used as control for each inoculum without any
106 substrate. The hydrogen production from the control was subtracted from the hydrogen
107 production obtained in the substrate assays prior to data analysis.

108 *2.4. Analyses*

109 Both the volume and composition of the biogas were determined daily. The
110 produced biogas was quantified using a gas flow meter (Ritter TG1) and a gas suction
111 pump (KNF Laboport). Gas volumes were converted to standard conditions and
112 corrected by subtracting the production of the blank. The composition of the biogas was
113 determined by gas chromatography separation (Shimadzu GC-2010 system). H_2, CO_2,

CH_4 and O_2 were analysed by means of a thermal conductivity detector (TCD) using a Supelco Carboxen 1010 Plot column [27]. Total solids (TS), volatile solids (VS), total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) were analysed according to the Standard Methods [28] at the beginning and end of each experiment. Volatile fatty acids (VFA) were determined by gas chromatography on a Shimadzu GC-2010 system equipped with a flame ionization detector (FID) and a capillary column filled with Nukol [29]. The pH was measured at the beginning and end of the tests using a Crison 20 Basic pH meter [28].

2.5. Microbial analyses

Fluorescence *in situ* hybridization (FISH) was used to count the microorganisms contained in the reactors. The main steps of FISH of whole cells using 16S rRNA-targeted oligonucleotide probes are cell fixation followed by permeabilization and hybridization with the desired probe(s). Samples from batch reactors were collected in sterile universal bottles at the beginning and end of the BHP test. A 1:1 (v/v) ratio of absolute ethanol was added to the bottles. The samples were stored at -20°C until they were fixed. Further details of this procedure are given in Montero et al. [30].

The technique used for fixing and permeabilizing cells was based on the method described by Amann et al. [31,32]. The 16S rRNA-targeted oligonucleotide probes used in this study are shown in Table 3: bacterial-universal probe EUB338 [31,32], and *Archaea*-universal probe ARC915 [33]. The cellular concentration and percentages of *Eubacteria* and *Archaea* were obtained by FISH. The total population was estimated as the sum of the populations of *Eubacteria* and *Archaea* for the reason that most anaerobic microorganisms in anaerobic reactors belong to these two groups [34]. Samples were examined visually and the cells were counted under an Axio Imager Upright epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp and a

100 x oil objective lens. The filter employed depended on the identity of the labelled probe: a B-2A filter (DM 510, Excitation 450-490 and Barrer 520) was used for 6-FAM; while a G-2A filter (DM 580, Excitation 510-560 and Barrer 590) was used for Cy3. In addition, microbial activity was evaluated from biochemical activity according to the methods reported by Montero et al. [30] and Zahedi et al. [35]. The activity was calculated as the ratio of H_2 generated and the number of microorganisms inside the reactor obtained by FISH staining.

Table 3. Oligonucleotide probes used in this study

	Probe sequences (from 5' to 3')	Target	Formamide(%)	Time (h)	T (°C)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	*Eubacteria*	20	1.5	46	[31,32]
ARC915	GTGCTCCCCCGCCAATTCCT	*Archaea*	35	1.5	46	[33]

3. Results and discussion

3.1. Physico-chemical analysis

The physical-chemical characteristics of three tests at the beginning and end of the tests are summarized in Table 4. The pH remained relatively stable during experimentation, varying from 5 to 5.5. There were no abrupt variations in pH, demonstrating that the systems were capable of self-regulating in order to favour microbial activity [34].

VS and TS removal rates ranged between 1.7 and 17.3%. The lowest rate was achieved with the acidogenic inoculum (Test 1).

As for SCOD removal, this was lower than 23% in all tests. Yang and Wang [2] also found that the SCOD concentration decreased, with significant reductions in removal rates of 7.1-31.3%. These authors state that their results indicated that the hydrolysis amount of particular organics by hydrolytic bacteria was lower than the

utilization amount of soluble organics by hydrogen producers. In terms of TCOD, the removal rate was greater, with percentages ranging between 50-60%. These results are in line with those obtained by Torquato et al. [21], in which the maximum removal rate of 41% was obtained in the digestion of vinasse to produce hydrogen. However, Silva et al. [17,18] reported that COD removal was lower than 20% when testing the co-digestion of food waste, sewage sludge and crude glycerol.

Table 4. Physico-chemical and microbial characterization of the three tests

Parameters	Units	Test 1		Test 2		Test 3	
		Initial	Final	Initial	Final	Initial	Final
Physico-chemical characteristics							
pH		5.35	5.07	5.32	5.27	5.46	5.39
TS	g/L	34.99	34.35	41.24	35.80	40.54	34.26
VS	g/L	27.67	27.19	32.25	27.83	31.33	25.90
TCOD	g/L	68.00	33.78	65.63	28.35	86.38	30.64
SCOD	g/L	35.38	27.17	26.44	22.46	25.75	22.83
Total VFA	g/L	3.40	5.31	2.53	5.27	2.80	5.90
Microbial characterization							
Total population	10^8cells/mL	13.51	13.29	14.95	85.90	15.29	13.27
Eubacteria	%	41.3	42.6	59.2	92.1	46.3	44.6
Archaea	%	58.7	57.5	40.8	8.0	53.7	55.4

As regards intermediate compounds, a large amount of VFAs was produced during the tests. At the end of the BHP tests, the dominant species were acetic, butyric and propionic acids, the concentrations for each inoculum being shown in Fig.1. Generally, hydrogen production via dark fermentation produces acetic and butyric acids as by-products [36]. Butyric acid was predominant in Test 2 (using SI), which presents a higher hydrogen yield. Luo et al. [37]and Chen et al. [38] also found that the highest

hydrogen production was obtained when butyric acid predominated. Butyric acid-type fermentation is considered one of the most effective pathways for hydrogen production [17]. On the other hand, TSI showed the highest production of propionic acid, which is detrimental for hydrogen production [19]. Tyagi et al. [3] found that hydrogen yield decreases with increasing propionic acid concentration.

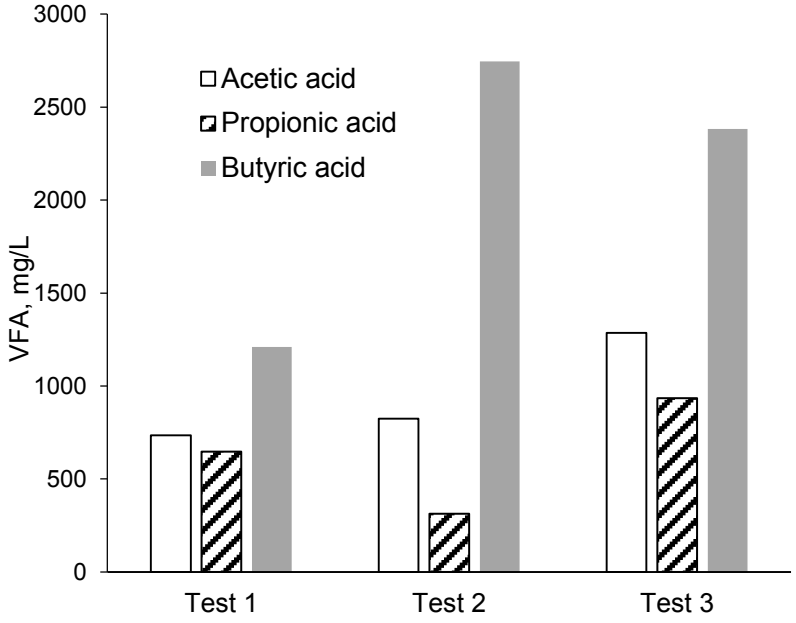
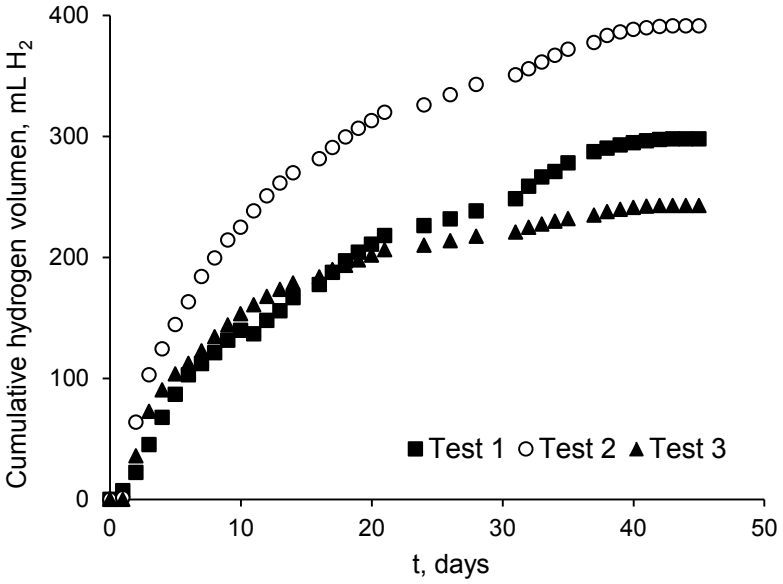


Figure 1. Volatile fatty acids generated during the tests.

3.2.Biogas production

Fig. 2 shows the cumulative hydrogen production for sludge-vinasse co-digestion with different inocula. In all the BHP tests, hydrogen production commenced in the first hours, as the lag phase was short. Furthermore, the biogas generated in all three tests was composed of hydrogen and carbon dioxide, no methanogenic activity being observed (i.e. the biogas was methane free). All this is due to the fact that the pH values fell within the 5-6 range, which is optimal to enhance H_2 generation and avoid methanogenesis [18].



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192 Figure 2. Cumulative hydrogen production during the operating of batch reactors with
193 different inocula.

194 The sludge inoculum led to the highest maximum accumulated H_2 volume
195 (391mL H_2) compared to the acidogenic inoculum (298mL H_2) and the thermally pre-
196 treated inoculum (TSI) (243mL H_2). In terms of H_2 yield (as per millilitres of hydrogen
197 per gram of volatile solids of the substrate initially added to each reactor), the highest
198 value was also achieved at the test using the SI (177mL H_2/g VS_{added}), corresponding to
199 an increase of 21 and 36% in relation to that obtained in the Test 1 (146 mL H_2/g
200 VS_{added}) and the Test 3 (130 mL H_2/g VS_{added}) (Fig.3). According to these results,
201 hydrogen production is inhibited rather than enhanced when the inoculum is submitted
202 to a thermal pre-treatment with the purpose of inactivating H_2-consuming *Archaea* and
203 avoiding methane generation, as proposed by several authors [2,17–23,25]. These
204 results are concordant and discrepant at the same time with those collected in the
205 literature. Thus, Luo et al. [37] also observed this tendency, the best condition was
206 without any inoculum treatment. However, Albanez et al. [39] observed a slight

improvement was noticed when performing the inoculum heat shock pretreatment in the co-digestion of vinasse and molasses. In a recent study, Lovato et al. [19]subjected the inoculum used in the co-digestion of cheese whey and glycerin to a heat shock pre-treatment (90°C for 10 min), obtaining significantly higher values for hydrogen productivities and yields than using untreated inoculum. In other studies using the same inoculum though treating glycerin-based wastewater, the thermally pre-treated inoculum was not found to be significantly different from the untreated sludge in terms of molar productivity and molar hydrogen yield [23]. It is important to emphasize that the last three studies were done in AnSBBR at mesophilic conditions.

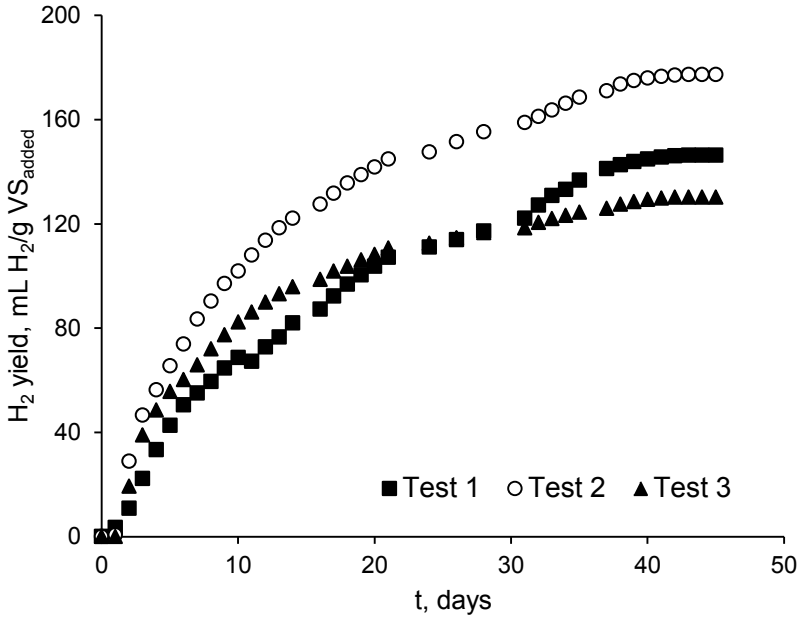
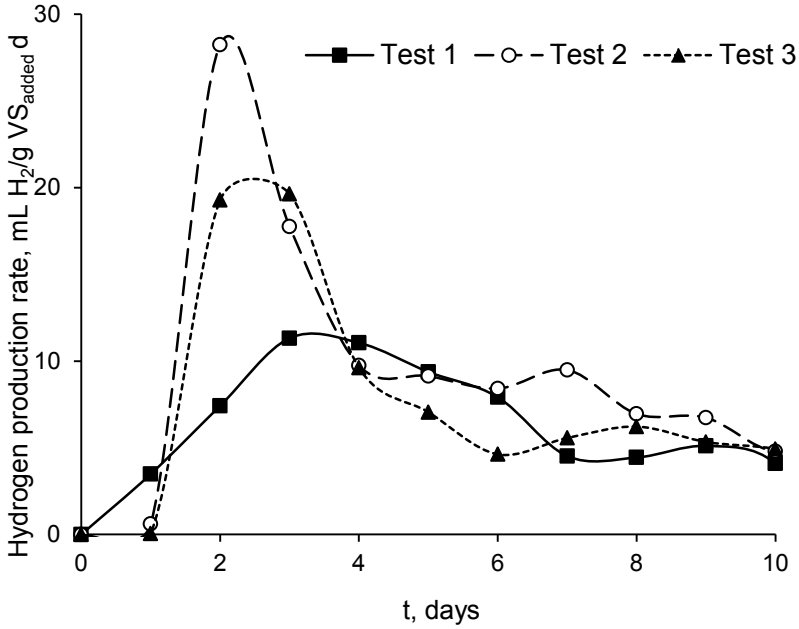


Figure 3. Hydrogen yield for batch tests using different inocula.

3.3.Hydrogen production rate

In order to ease identification of differences between the inocula, the hydrogen production rate of the first ten days is shown in Fig. 4. As for the SI and STI inocula

222 behaved similarly with a significant lead of SI inoculum. This could be expected
223 because both inocula have the same source. As for AI inoculum, a broader and lower
224 peak than in the other inocula was detected. The maximum hydrogen production rate
225 observed in the tests 1 and 2, with the AI and STI inocula, reached the peak after three
226 days, and amounted to 11 mL H_2/(gVS_{added} d) and 20 mL H_2/(gVS_{added} d), respectively.
227 On the other hand, the maximum hydrogen production rate observed in the Test 2 with
228 the sludge inoculum reached the peak at about the second day of experimentation,
229 amounting to 28 mL H_2/(gVS_{added} d). As could be expected, the highest maximum
230 hydrogen production rate was noticed in the experiment with higher hydrogen yield.



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232 Figure 4. Hydrogen production rate for batch tests using different inocula.

233 *3.4. Statistical analysis*

234 Fig. 5 shows the average of hydrogen yield produce to each inoculum with their
235 standard deviation. In order to evaluate differences between results of the three inocula,
236 hydrogen yield and hydrogen production rate results were analysed statistically by

single-factor analysis of variance (ANOVA). Table 5 shows the results of this analysis. A confidence level of 95% was selected for all comparisons.

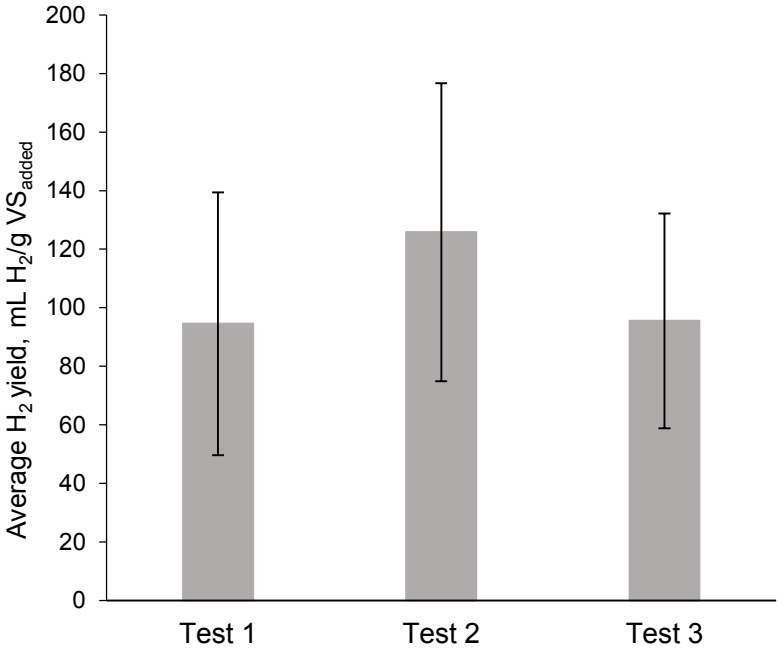

Figure 5. Average hydrogen yield for batch tests using different inocula with standard deviation.

In the matter of hydrogen yield, for the comparison between inocula, the p value is smaller than 0.05 in all cases, therefore there is significant difference between the yields of hydrogen produced in SI inoculum and those of the other two.

Table 5. ANOVA results for the hydrogen yield and hydrogen production rate.

		Degrees of freedom	Sum of squares	Mean square	F value	P value
Hydrogen yield	Inoculas AI and SI	1	18559	18559	8.057	0.00585
	Inoculas SI and STI	1	17435	17435	8.865	0.00393
	Inoculas AI and STI	1	18	17.6	0.01	0.919
Hydrogen production rate	Inoculas AI and SI	1	12.5	12.53	0.691	0.409
	Inoculas SI and STI	1	29.1	29.13	1.238	0.269
	Inoculas AI and STI	1	3.5	3.452	0.249	0.619

Conversely, for the hydrogen production rate there is no significant difference ($p > 0.05$) between all of the three tested inocula.

3.5. Microbial population dynamics

The concentrations of microorganisms in the samples before and after the different tests were studied. The amounts and relative percentages of the main microbial groups are shown in Table 4. In Test 2, in which the highest hydrogen yield (177mL H_2/g VS_{added}) was obtained, the population size increased during the time of experimentation. Instead, in Test 1 and Test 3, the population size remained stable at the end of the BHP tests in all cases; significantly, the amount of substrate for acidogenic phase was sufficient. *Eubacteria* was the major phylogenetic domain in all cases. No significant variation was found in *Eubacteria: Archaea* ratios at the beginning and end of the experiments in Test 1 or Test 3: 41-46% and 59-54%, respectively. In Test 2, however, the percentages of *Eubacteria* increased from 59% to 92%. Thus, BHP test with sludge inoculum could increase the abundance of the specific bacteria in the reactor, which were beneficial for the hydrogen production.

Although methane is not generated, the analyses showed the largest number of *Archaea* present. In terms of productivity, it may be stated that *Archaea* were inactive [40].

4. Conclusions

H_2 generation from sludge vinasse co-digestion, using different inocula, was studied. The batch tests were successfully in all cases. Significant differences have been found in the production of hydrogen among the three inoculums. The highest hydrogen yield,177mL H_2/g VS_{added}, was obtained with a sludge inoculum. Even though, Eubacteria was the major phylogenetic domain in all cases, sludge inoculum showed a greater growth of Eubacteria during the test, increasing the percentage of this population from 59.2 to 92.1. The rate of hydrogen production was comparable between the

different inocula, that is, the duration of the test is independent of the type of inoculum used. Furthermore, hydrogen production was chiefly accompanied by the production of acetic and butyric acids.

Acknowledgements

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Inoculum			Substrates	Experimentation conditions	Maximum hydrogen yield	References
Type	Source	Pretratament				
Anaerobic sludge	Wastewater	100°C, 15 min	Sludge and perennial ryegrass	Batch 37°C	60mL H_2/g VS_{added}	[2]
Anaerobic sludge	Municipal sewage	100°C, 30 min	Food waste and crude glycerol	Batch 35°C	180mL H_2/g $VS_{consumed}$	[17]
Anaerobic sludge	Municipal sewage	100°C, 30 min	Food waste, sewage sludge and crude glycerol	Batch 35°C	179mL H_2/g $VS_{consumed}$	[18]
Anaerobic sludge	OFMSW		OFMSW and sewage sludge	Batch 55°C	51mL H_2/g $VS_{consumed}$	[3]
Anaerobic sludge (Upflow anaerobic sludge blanket UASB)	Wastewater from poultry slaughterhouse	90°C, 10 min	Cheese whey and glycerin	Anaerobic sequencing batch biofilm reactor (AnSBBR) 30°C	5.4mol H_2/kg COD 2.3mol H_2/kg COD	[19]
Granular (UASB)	Wastewater from poultry slaughterhouse	90°C, 10 min	Glycerin-based wastewater	AnSBBR 30°C	20mol H_2/kg $COD_{consumed}$ 19.8mol H_2/kg $COD_{consumed}$	[23]
Granular mesophilic sludge (UASB)	Potato wastes	105°C, 4h	Glucose Wheat bran from common wheat Wheat bran from durum wheat Wastes from mashed	Batch 35°C	185L H_2/kg COD 47L H_2/kg COD 76L H_2/kg COD 177L H_2/kg	[25]

			potatoes		COD	
			Wastes from steam-peeling potatoes		134L H_2/kg COD	
Anaerobic granular sludge	Municipal sewage	100°C, 10 min	Citrus vinasse	Batch 37°C	2.0mmol H_2/g COD	[21]
Anaerobic sludge	Citrate-producing wastewater	102°C, 30 min	Brewery wastewater	Batch 36°C	149.6mL H_2/g COD	[24]
Anaerobic (Fixed-bed)	Sucrose-based synthetic wastewater		Domestic sewage	Batch 25°C	6.01mmol H_2/g COD	[26]
			Glycerin wastewater		6.03mmol H_2/g COD	
			Sugarcane vinasse		24.97mmol H_2/g COD	
Anaerobic (Fixed-bed)	Sucrose-based synthetic wastewater		Vinasse	Batch 25°C	20.8mL H_2/g COD	[12]
Anaerobic mixed microflora (UASB)	Chemical wastewater	100°C, 2h pH 3, 24h	Synthetic wastewater and domestic sewage wastewater	Batch 29°C	0.71mmol H_2/g COD	[22]
Anaerobic sludge (UASB)	Wastewater from poultry slaughterhouse	90°C, 10 min	Sugarcane vinasse	Batch 55°C	2.31mmol H_2/g COD	[20]
Anaerobic granular sludge		90°C, 1 h	Cassava stillage	Batch 60°C	65.3mL H_2/g VS	[37]
		Chloroform 0.2%			57.4mL H_2/g VS	
		pH 12			32.9mL H_2/g VS	
		pH 3			59.0mL H_2/g VS	
		Loading-shock			46.5mL H_2/g VS	
					64.4mL H_2/g VS	
Anaerobic sludge	Wastewater from poultry slaughterhouse	90°C, 15 min	Vinasse and molasses	AnSBBR	0.8mol H_2/kg $COD_{consumed}$	[39]
					0.5mol H_2/kg $COD_{consumed}$	
Anaerobic sludge	Waste activated sludge and vinasse		Waste activated sludge and vinasse	Batch 55°C	146.37mL H_2/g Vs_{added}	The present study
	Waste activated sludge				177.23mL H_2/g VS_{added}	
	Waste activated sludge	100°C, 15 min			130.17mL H_2/g VS_{added}	

423

424 Table 1. Comparative study on hydrogen production in anaerobic reactors.